Association between a common missense variant in LOXL3 gene and the risk of non-syndromic cleft palate

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ABSTRACT

To investigate possible association between functional common variants in the lysyl oxidase like 3 (*LOXL3*) gene and non-syndromic cleft palate (nsCP) we selected a common missense variant p.Ile615Phe (rs17010021), which was predicted to have a probably damaging effect on the *LOXL3* enzyme. We genotyped 258 nsCP case-parent triads of European origin and tested genetic association using the transmission disequilibrium test (TDT) and log-linear regression analyses of genotypic relative risks (RR) and of parent-of-origin effects. The observed genotype frequency in parents was in Hardy-Weinberg equilibrium. Compared with wild-type Ile/Ile homozygotes, the RR for Phe/Phe homozygote infants was 6.87 (p-value $3.0x10^{-3}$), while that for Ile/Phe heterozygotes was not significant. Assuming an autosomal recessive model, the RR for Phe/Phe genotype resulted 10.54 (p-value $2.9x10^{-5}$), with a 3.6% population attributable risk. No parental-of-origin effect was observed. The identification in *LOXL3* of a missense variant which under a recessive model associates with ten-fold increased risk of nsCP supports the hypothesis that the genetic etiology of this congenital anomaly includes relatively uncommon recessive variants with moderate penetrance and located in genes which are also involved in syndromes that include CP as part of the phenotype. Our findings require functional validation and replication in a larger independent genetic association study.

Key Words: lysyl oxidase like 3, non-syndromic, cleft palate, missense variant.

INTRODUCTION

The programming of palatal development starts early in the 4th week, with the formation of facial primordia that involves a complex series of closely coordinated events that includes proliferation, differentiation and morphogenetic movement (Dixon et al. 2011; Mossey et al. 2009). By the end of the 6th week, the primary palate is formed by the fusion of the medial nasal process with the maxillary process. The secondary palate arises as bilateral, medially directed outgrowths of the maxillary processes (palatal shelves) that initially grows vertically on either side of the tongue but later elevate to a horizontal position above the tongue. This horizontal growth of the adjacent palatal shelves leads to their contact with one another and fusion to form the secondary palate (Dixon et al. 2011). Disruption or perturbations at any step during this process that includes elevation, migration or fusion is likely to induce cleft palate.

Cleft palate (CP) is a common congenital orofacial malformation. Its prevalence at birth varies with geography and ethnicity between 1 and 25 per 10,000 live births, highest in non-Hispanic Whites and lowest in Africans (Burg et al. 2016). The sex ratio (male:female) of CP is 1:2. This might possibly be explained by differential gene expression as observed between sexes in animal models (Suazo et al. 2011), differential effects of female hormones (Miura et al. 1990), or delayed fusion of palate in females (Burg et al. 2016). Approximately 50% of CP cases are non-syndromic (nsCP), (Mai et al. 2014; Watkins et al. 2014), and are generally considered multifactorial conditions, due to interplay between genetic and environmental factors (Dixon et al. 2011; Mangold et al. 2011).

nsCP shows strong familial aggregation, which suggests a genetic component to etiology (Marazita & Leslie 2016). Analyses of nationwide records from Norway and Denmark show an increase in risk of recurrence among first-degree relatives of affected individuals (Sivertsen et al. 2008; Grosen et al. 2010). The environmental factors contributing to CP etiology so far identified are largely the same as those for cleft of the lip and palate (CL/P) including tobacco smoke and alcohol (Little et al. 2004; Sabbagh et al. 2015; Bell et al. 2014) and an inverse association with reported maternal use of vitamin supplements (Butali et al. 2013).

A number of genetic factors associated with non-syndromic oral clefts have been identified, mainly for CL/P rather than CP. Based on these genetic findings, non-syndromic CL/P and non-syndromic CP are considered to have only very limited overlap in terms of their genetic etiology (Cura et al. 2016). This is further supported by genome-wide association studies (GWASs) or meta-analyses of GWAS data in different populations that have identified 37 risk loci for non-syndromic CL/P (Birnbaum et al. 2009; Mangold et al. 2010; Beaty et al. 2010, 2013; Leslie et al. 2015, 2017; Yu et al. 2017; Ludwig et al. 2017; Ludwig et al. 2017; Ishorst et al., 2018), but just one replicated finding for non-syndromic CP (Leslie et al. 2016; Mangold et al. 2016).

Although GWAS have helped detect and replicate associations between common gene variants and orofacial clefts, the proportion of heritability accounted for by these variants is relatively low, with inconsistencies across studies (Beaty et al. 2016). GWAS have typically been designed to minimize the risk of false positive genetic associations in common chronic disease, but it is known that there is a substantial risk of false negatives (Ioannidis et al. 2011). Moreover, it has been observed that common variants

of genes that are involved in Mendelian disorders have been associated with non-Mendelian forms of the same disorders (Blair et al. 2013). In addition, variants of some genes involved in syndromic clefts have been found to have replicated associations with non-syndromic clefts, reflecting the two forms of clefting as parts of a single spectrum (Stanier & Moore 2004; Dixon et al. 2011). An excellent example is *GRHL3*, the second gene associated with Van der Woude syndrome and its recent identification as associated with nsCP (Leslie et al. 2016; Mangold et al. 2016).

The human *LOXL3* gene located on chromosome 2p13.1 has been associated with Stickler syndrome (MIM #108300), which includes CP as a phenotype (Alzahrani et al. 2015). Additionally, deletion of this gene impairs collagen assembly and crosslinking during palate development in mouse model (Zhang et al. 2015). However, no evidence of linkage or association with nsCP was reported for this gene in a recent GWAS (Leslie et al. 2016) or an imputation based meta-analysis of GWAS data (Ludwig et al. 2017). Of note, the ability to detect common variants with very weak effects, or less common variants with small to modest effects, is strongly dependent on assumptions concerning linkage disequilibrium, allele frequency and genotype certainty (Bomba et al. 2017).

We therefore examined the potential association between putative functional variants of *LOXL3* and nsCP, a disorder that is less common than other nsCL/P in humans, and less investigated, in European case-parent triads.

MATERIALS AND METHODS

Participants

The study includes 258 nuclear families of infants with nsCP identified through the EUROCRAN and ITALCLEFT biobanks, which include case-parent trios from 9 European countries (Mossey et al. 2017; Ghassibe-Sabbagh et al. 2011), including the United Kingdom, Netherlands, Italy, Spain, Slovenia, Slovakia, Hungary, Estonia and Bulgaria. The case-parent trio design of the present study makes it less vulnerable to population stratification, a particular concern of multi-centre studies [Mossey et al. 2017]. Ethical permission was sought and obtained at surgical centres in each participating countries at the time of first surgical intervention on the index infant. Infants with recognized syndromic clefts or Pierre Robin sequence were excluded. Peripheral blood or buccal cell samples were used to obtain genomic DNA from infants and their parents. The use of data and DNA samples from EUROCRAN and ITALCLEFT biobanks was approved by MREC Scotland (Dec 7th 2011, #MREC/1/0/7) and S. Paolo Hosp. E.C. (Mar 2nd, 2012, #3503) respectively.

Exposure information

In both the EUROCRAN and ITALCLEFT studies mothers were asked to respond to a specific questionnaire that was administered by personal interview when the index affected infant was brought in to the surgical centre to undergo the primary surgery. Major areas about which information was sought included use of nutritional supplements and tobacco smoking. Folic acid supplementation was defined as having taken folic acid or folic acid-containing supplements (at least 0.4 mg/day) for at least one month during the periconceptional period (3 months before to 3 months after conception). Maternal smoking during pregnancy was defined as having smoked at least one cigarette per day during the periconceptional period (Mossey et al. 2017).

Selection of putative functional single nucleotide variants in *LOXL3*

We screened the exons of the *LOXL3* gene for nucleotide substitutions and insertions and deletions using the UCSC Genome browser GRCh38/hg38 assembly (https://genome-euro.ucsc.edu/) and identified 336 missense and 139 synonymous variants. Of these, according to dbSNP build 150 (www.ncbi.nlm.nih.gov/projects/SNP), only three are polymorphic, with minor allele frequency (MAF) >1%: rs17010022, rs17010021, and rs77706750. The first SNP, rs17010022, is a synonymous p.Leu371Leu variant located in exon 7 of *LOXL3* gene, with putative no effect on conformation of the encoded peptide, and therefore was discarded. The other two variants, rs77706750 in exon 7, and rs17010021 in exon 11, cause substitutions (p.Arg375His and p.Ile615Phe, respectively) both predicted to be "probably damaging" by PolyPhen-2 (Adzhubei et al. 2013). However, considering the available sample size, the MAF of rs77706750 was too low (1.46%) to provide enough power (0.80) under dominant or recessive genetic models (Quanto 1.2.4, biostats.usc.edu), and hence was not included in the present study. However, the MAF of rs17010021 was much higher (8.23% reported in dbSNP), granting sufficient power for a genetic association study.

Genotyping

For most individuals included in the study, genomic DNA (gDNA) was extracted from peripheral blood specimens using the Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH). For around 5% of participants, gDNA was extracted from buccal swab specimens using QIAamp DNA Blood Mini Kit (Qiagen, Hilden DE) according to the manufacturer's instructions. All gDNA samples were quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA).

Genotypes of p.Ile615Phe variant were obtained by TaqMan allelic discrimination assay using an ABI 7300 real-time thermocycler according to the standard protocol of manufacturer (Applied BioSystems, Foster City, CA). In 15% of samples, genotyping was repeated for quality testing.

Statistical analysis

The $\chi 2$ test for the Hardy-Weinberg equilibrium (HWE) were computed for genotypes of parents and case-infants. The genetic association of the missense variant in nsCP case-parent triads was calculated using the transmission disequilibrium test (TDT), (Spielman et al. 1993). We estimated relative risk (RR) and 95% of confidence interval (CI) for the independent effects of mother and infant genotypes using a log-linear regression model that incorporates an expectation-maximization algorithm to allow inclusion of triads for which both parent genotype were missing (Weinberg et al. 1998; Wilcox et al. 1998). The analyses were implemented using the Stata package (http://www.biostat-resources.com, StataCorp LP, College Station, TX). As exploratory analyses, we carried out subgroup analyses stratifying on the sex of the infant and maternal smoking and use of supplements containing folic acid.

We further investigated a possible parent-of-origin effect, by assessing the risk increment (I_M) in the offspring associated with receiving the allele transmitted from the mother as compared to the father in log-linear regression analysis (Weinberg et al. 1998; Wilcox et al. 1998).

RESULTS

198 The study included 258 nsCP case-parent trios from 9 European countries. As expected,
199 female cases outnumbered the males, and male:female sex ratio was 0.78 (95% C.I.
200 0.74-0.84).

201 The allele and genotype frequency of the triads included in the study is shown in Table
202 1. Among the 516 parents included in the study the frequency of Phe allele was 4.7%
203 (95%CI 2.9-6.5%), a value lower than the 8.23% reported in dbSNP. Genotype
204 frequency among cases was significantly out of Hardy-Weinberg equilibrium (p-value =
205 $2.27x10^{-6}$), while both parents resulted not in disequilibrium (p-value = 0.68).
206 Remarkably, the frequency of Phe/Phe homozygotes, predicted to be only 0.22% on the
207 basis of allele frequency in the parents, was 7-fold higher (1.55%) than predicted among
208 nsCP cases.

209 Application of TDT showed no significant evidence of asymmetric segregation of Phe
210 allele from parents (Transmitted:Non-transmitted = 21:23, p-value = 0.673).
211 Considering the observed low frequency of Ile/Phe genotype among parents (Table 1),
212 and being the power of the TDT heavily dependent on the number of heterozygous
213 parents (Sebro, Rogus, 2010), we performed the calculation of genotype-associated RR
214 using a log-linear regression model (Weinberg et al. 1998; Wilcox et al. 1998).
215 Calculation of genotype-associated RR showed significant association between Phe/Phe
216 homozygous infant genotype and nsCP risk (RR = 6.9, p-value = 0.003), whereas there
217 was no significant association with the heterozygous genotype. Mother's genotype was
218 not associated with increased risk of nsCP in the offspring (Table 2).

219 Considering that the Ile/Phe genotype provided no increased risk of nsCP compared to
220 wild type Ile/Ile homozygotes, while Phe/Phe genotype associated with increased risk,

we assumed a recessive genetic model. Under this model, log-linear regression analysis showed that infant's Phe/Phe genotype associated with a significant ten-fold increased risk of nsCP (RR = 10.54 (95% C.I. 3.34-33.30, p-value = $2.85x10^{-5}$). No parental of origin effect was observed (I^M = 0.58, p-value = 0.455).

Considering the genotypic frequencies of parents as reference and a birth prevalence of nsCP of 1:2216 among Europeans (Calzolari et al., 2004), the population attributable risk of Phe/Phe genotype was 3.6%, whereas the penetrance was 0.48%.

Although we are aware of the limited sample size of our study, we conducted subgroup analyses and report these. Among the four Phe/Phe infants, three females and one male, only one girl was born from a mother exposed to folic acid supplementation during the periconceptional period. As regards periconceptional exposure to tobacco smoking, all four Phe/Phe infants were born from non-smoking mothers. RR did not significantly differed between male and female cases.

DISCUSSION

In the present study, we investigated a potential association between functional common variants in lysyl oxidase like 3 (*LOXL3)* gene and the risk of developing nsCP. Rare variants in *LOXL3* have been detected in patients with Stickler syndrome, which may present with CP (Alzahrani et al. 2015), and in mouse model a crucial role of Loxl3 gene in palate development has been demonstrated (Zhang et al. 2015). Among the hundreds of missense variants annotated in *LOXL3* gene we selected p.Ile615Phe, which is the only one that is predicted to be probably damaging and has relatively high MAF, sufficient to provide enough statistical power considering the sample size of the study.

Although Phe/Phe homozygotes are very uncommon, we identified four Phe/Phe homozygotes among the 258 cases included in the study, and detected a significant association between infant's homozygote Phe/Phe genotype and the risk of nsCP, compared to common Ile/Ile homozygotes. Heterozygous Ile/Phe genotype was not significantly associate with nsCP. Therefore, assuming an autosomal recessive model, the Phe/Phe genotype turned out to associate with around ten-fold increased risk of nsCP (p-value = 2.85×10^{-5}). Autosomal recessive genetic model is typical for enzyme-encoding genes, and fits well with the nature of *LOXL3*. As the p.615Phe enzyme is predicted to have lost most or all catalytic activity, we presume that Phe/Phe homozygotes are severely deficient of the amine oxidase activity of *LOXL3* enzyme, and consequently have impaired collagen fiber assembly in palatal mesenchyme. We hypothesize that this impairment could have played a role in determining the failure of fusion of palatal shelves during embryogenesis, and ultimately caused CP. The lack of efficient catalysis of collagen crosslinking associated with p.615Phe enzyme may resemble the effect of LOX's inhibitor β-aminopropionitrile, which determine reduced collagen fibres density and development of CP in animal model (Pratt & King 1972). Functional studies using animal models are awaited to confirm the phenotypic effect of p.615Phe enzyme.

The failure of TDT to detect association could rely on the fact that, due to the relative low MAF of the studied *LOXL3* variant, among the cases most of p.615Phe alleles are carried by heterozygotes, which are not at risk of nsCP, and therefore distortion of transmission from parents would not be expected.

As might be anticipated for a gene expressed in palate shelves during embryonic development, maternal p.Ile615Phe genotype was not associated with the infant's risk of nsCP. Moreover, no preferential transmission of minor allele from one of two parents was observed. Due to the low frequency of Phe/Phe homozygotes, the statistical power of the study was not sufficient to detect interaction with infant sex, periconceptional folic acid supplementation, or exposure to tobacco smoking.

The infant's Phe/Phe genotype seems to strongly increase the risk of nsCP, but its actual weight among the multiple genetic and environmental factors as part of the multifactorial etiology of nsCP is relatively small. Due to the relatively low MAF of p.Ile615Phe, the calculated population attributable risk was only 3.6%, and the penetrance modest (0.48%). We hypothesize that other functional variants of the *LOXL3* gene, mainly classified as rare variants and less frequent than the p.Ile615Phe variant, might be associated with nsCP risk.

The impact of rare or less common variants associated with increased risk of nsCP has begun to emerge from recent exome-wide and genome-wide sequencing studies (Mangold et al. 2016). In particular, a low frequency missense p.Thr454Met variant in *GRHL3* (rs41268753) was significantly associated with nsCP risk (Mangold et al. 2016; Leslie et al. 2016). From the latest genetic investigations on nsCP, a difference in terms of frequency spectrum of susceptibility variants compared to nsCL/P, is becoming evident. While GWAS of nsCL/P identified a number of common polymorphic variants (Birnbaum et al. 2009; Beaty et al. 2010; Mangold et al. 2010), GWAS of nsCP have detected only one genome-wide significant variant (Leslie et al. 2016), even though sample size were comparable. This evidence suggests that the genetic aetiology of nsCP

may mainly rely on relatively rare variants, or less common variants that act under recessive model, which may present moderate penetrance, tending to escape detection by genome-wide studies and to be located within genes involved in syndromes that include CP as part of the phenotype. *LOXL3* p.Ile615Phe may be one of these variants.

In conclusion, using a candidate gene approach, we identified a missense variant in *LOXL3* gene, p.Ile615Phe, which under a recessive model is associated with a significant ten-fold increased risk of nsCP. This finding should be replicated in a larger cohort of case-parent trios, and joint effects with environmental exposure factors investigated. We suggest that *LOXL3* p.Ile615Phe, along with *GRHL3* p.Thr454Met, are part of a constellation of low frequency variants that compose the genetic background of nsCP.

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434

435 **Table 1** Allele and genotype frequencies of p.Ile615Phe (rs17010021) in 258
436 nsCP case-parent triads, and p-value of difference from Hardy-Weinberg (H-W)
437 equilibrium.

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Alleles/Genotypes	Cases n (%)	Mothers n (%)	Fathers n (%)
Ile	493 (95.5)	498 (96.5)	486 (94.2)
Phe	23 (4.5)	18 (3.5)	30 (5.8)
Ile/Ile	239 (92.6)	240 (93.0)	230 (89.1)
Ile/Phe	15 (5.8)	18 (7.0)	26(10.1)
Phe/Phe	4 (1.6)	0 (0.0)	2 (0.8)
H-W p-value	2.27×10^{-6}	0.85	0.44

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Table 2 Genotype-associated relative risk of p.Ile615Phe (rs17010021) in 258 nsCP case-parent triads assuming the common Ile/Ile homozygous genotype as reference.

Mother's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.54 (0.28-1.05)	0.071
Phe/Phe	n.c.	-
Infant's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.61 (0.31-1.17)	0.136
Phe/Phe	6.87 (1.97-23.98)	0.003